**Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
28th Report
(Study period: December 1ˢᵗ to December 7ᵗʰ, 2023)**

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Main findings:

- During the week of 1 December to 7 December 2023, a total of 98 wastewater samples were collected from 15 Regions and 2 Autonomous Province (A.P.).
- Mutations characteristic of the Omicron variant were identified in 14 of these Regions/AP, while no sequencing data were collected from the remaining areas.
- Analysis of the sequences obtained by Sanger sequencing showed that 67% of the positive samples displayed amino acid substitutions that are associated to the Omicron XBB.1.5/1.9* lineages, while 30% of the sampleshowed substitutions of the Omicron JN.1* lineage. In addition, 3% displayed mutations of the Omicron XBB.1.16* lineage.

Introduction

On March 17th, 2021, the European Union Commission issued Recommendation 2021/472, encouraging Member States to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewater by October 1st, 2021. In response to this recommendation, the Istituto Superiore di Sanità (ISS) started a series of "flash surveys". These surveys consist of monthly sampling campaigns carried out over short periods in different locations throughout Italy. The primary objective of these flash surveys is to gather supplementary information on SARS-CoV-2 variants in the population, complementing data obtained through clinical surveillance. The aim of this report is to summarise the results of the 28th national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 1 to 7 December 2023.

Methodology

The 28th national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out from 1 to 7 December 2023. The survey involved 98 sewage samples from 86 wastewater treatment plants (WTPs) located across 15 Regions and 2 Autonomous Provinces. Information on the WTPs participating in the SARS-CoV-2 surveillance in urban wastewater in Italy can be found on the ISS website[1]. The samples collected during the survey were processed and the viral concentration was determined by laboratories within the SARI network using the protocol "Sorveglianza di SARS-CoV-2 in reflui urbani - Protocollo progetto SARI - rev.3"[2]. Purified RNA extracts from the samples were delivered to ISS for variant detection.

For sequencing purposes, we employed a long-nested PCR assay covering approximately 1330 base pairs and spanning from amino acid residues 34 to 475 of the spike protein (PCR ID 1033/1034). After amplifying the target sequences, we used Sanger sequencing on individual samples.

For variant classification, we adopted the lineage classification based on 'outbreak.info'[3]

Results

Real Time qPCR

Out of the 98 samples analysed, a total of 95 (97%) tested positive for SARS-CoV-2 using the real-time RT-qPCR method employed for environmental surveillance (Table 1). The viral

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1 Surveillance of SARS-CoV-2 in urban wastewater in Italy 1° Report (Study period: 01 October 2021 - 31 March 2022) 8e5e2edb-bae0-f1b0-ee6e-08255c76484f (iss.it)

2 DOI 10.5281/zenodo.5758724.

3 https://outbreak.info/situation-reports, date: 05/12/2023

concentrations detected in these samples varied, ranging from 2.50E + 02 to 1.17E + 06 genome copies (g.c.) per liter of sewage.

Sanger Sequencing

Table 1 summarises the results of the long-nested PCR assay and sequencing methods. A total of 43 samples (45%) from 14 Regions/AP were successfully amplified. High-quality sequences were obtained by Sanger sequencing from 27 of these samples, while10 samples yielded only partial sequences were obtained due to high signal noise. In addition, in the case of 6 samples, sequence reading was hamperedby significant signal overlap (mixed electropherograms).
The wastewater analysis revealed the presence of five distinct SARS-CoV-2 lineages, as shown in Tables 1 and 2. Among these, the Omicron XBB.1.5/1.9* lineages (which are not distinguishable in the sequenced region) emerged as the predominant variant, accounting for 67% of the positive samples (25 samples). Following closely, the Omicron JN.1* lineage was detected in 30% of the positive samples (11 samples). In addition, the Omicron XBB.1.16* lineage was identified in 3% of the samples (one sample).
For ease of understanding, the mutations have been grouped into panels or "mutation packages". These are listed below:

- **Package A (assigned to the lineage Omicron XBB.1.5*/XBB.1.9*)** = V83A, G142D, DEL144, H146Q/H146K, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K
- **Package B (assigned to the lineage Omicron XBB.1.5*/XBB.1.9*)**[4] = Q52H, V83A, G142D, DEL144, H146Q, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, F456L, N460K
- **Package C (assigned to the Omicron XBB.1.16*)** = V83A, G142D, DEL144, H146Q, E180V, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K
- **Package D (assigned to the Omicron JN.1*)** = DEL69/70, V127F, G142D, DEL144, F157S, R158G, DEL211/212, V213G, L216F, H245N, A264D, I332V, G339H, K356T, S371F, S373P, S375F, T376A, R403K, D405N, R408S, K417N, N440K, V445H, G446S, N450D, L452W, L455S, N460K

The Omicron XBB.1.5*/XBB.1.9* lineages were detected across 10 regions/A.P., specifically Abruzzo, Basilicata, Emilia-Romagna, Friuli-Venezia-Giulia, Liguria, Molise, Puglia, Sicilia, Veneto and A.P. Bolzano. Instead, the Omicron JN.1* lineage was identified in 6 regions: Liguria, Marche, Emilia-Romagna, Umbria, Veneto and A.P. Trento. Key mutations of the Omicron XBB.1.16* lineage were detected solely within the Veneto region.

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[4] Package B may indicate the presence of the Omicron EG.5 sublineage. This last sublineage is characterised by the presence of the 'Q52H' mutation in approximately 93.2% and the 'F456L' mutation in about 96.2% of its sequences (https://outbreak.info/situation-reports, date: 26/01/2024).

Table 1. PCR and sequencing results

	Sample ID	Region/A.P.	City	WTP	RT-qPCR (c.g./L)	Mutations found by Sanger sequencing (long PCR ID_1034)	Sars-CoV-2 lineages (Sanger sequencing)
1	23863	Abruzzo	Chieti	S.Martino	1,35E+03	Package B[a] + F59S	Omicron XBB.1.5/1.9*
2	23864		Pescara	Via Raiale	1,13E+03	Package B[a] + F157L + L452R	Omicron XBB.1.5/1.9*
3	23866		L'Aquila	Pile	1,51E+03		
4	23867		Teramo	Villa Pavone	1,73E+03	M.E.[b]	Not assigned
5	23865		Pescara	Villa Carmine	1,07E+03		
6	23751	Basilicata	Potenza	Tiera di Vaglio	2,27E+05		
7	23752		Matera	Pantano	3,93E+05	Package B[a] + L455F	Omicron XBB.1.5/1.9*
8	23661	Emilia-Romagna	Ferrara	Ferrara - Linea 1	4,35E+05		
9	23662		Ferrara	Ferrara - Linea 2	<LOD		
10	23663		Modena	Carpi	6,10E+04		
11	23719		Piacenza	Borgoforte	3,65E+04		
12	23720		Parma	Parma ovest	1,40E+05		
13	23721		Reggio Emilia	Mancasale	1,52E+05		
14	23806		Modena	Naviglio	2,15E+05	Package B[a]	Omicron XBB.1.5/1.9*
15	23807		Ravenna - Forlì-Cesena	Ravenna	1,87E+05		
16	23812		Bologna	Imola	6,00E+04	Package B[a] + L455F	Omicron XBB.1.5/1.9*
17	23813		Ravenna	Faenza	3,43E+04	M.E.[b]	Not assigned
18	23814		Bologna	IDAR	9,23E+04	Package D (partial sequence, from V213G to N460K) [c]	Omicron JN.1*
19	23815		Forlì-Cesena	Forlì	9,88E+04	Package B[a] + L455F	Omicron XBB.1.5/1.9*
20	23816		Forlì-Cesena	Cesena	1,81E+05		
81	23817		Rimini - Forlì-Cesena	S. Giustina	1,32E+05	Package D (partial sequence, from H245N to N460K) [c]	Omicron JN.1*
78	23829	Friuli-Venezia-Giulia	Servola	Trieste	1,09E+05	Package B[a] + L455F	Omicron XBB.1.5/1.9*
79	23828		Cordenons	Pordenone	2,45E+04	M.E.[b]	Not assigned
80	23830		Udine	Udine	9,68E+04		

21	23639	Lazio	Roma	Civitavecchia Fiumaretta	2,50E+02	M.E.[b]	Not assigned
22	23676		Savona	Savona	2,01E+05	Package B[a] + L452R (partial sequence including Q52H and from G252V to N460K)[c]	Omicron XBB.1.5/1.9*
23	23677		Genova	Pegli	2,89E+05	Package D + N394K	Omicron JN.1*
24	23678		Genova	Voltri	1,09E+05	Package D (partial from L216F to N460K)[c]	Omicron JN.1*
25	23679		Genova	Quinto	1,00E+05	Package B[a] + F157L	Omicron XBB.1.5/1.9*
26	23680		Genova	Rapallo	1,48E+05	Package D (partial sequence, from A264D to N460K)[c]	Omicron JN.1*
27	23681		Genova	Sestri P	3,67E+04	Package D	Omicron JN.1*
28	23682	Liguria	Genova	Sturla	1,67E+05	Package A (partial sequence, from V213E to N460K)[c] + F456L	Omicron XBB.1.5/1.9*
29	23683		Genova	Darsena	1,20E+05	Package B[a]	Omicron XBB.1.5/1.9*
30	23684		Genova	Punta Vagno Genova	4,28E+05	Package D	Omicron JN.1*
31	23685		Genova	Valpolcevera	3,17E+04		
33	23687		Imperia	Sanremo - località Capo Verde	3,11E+04	Package A + L455F + F456L	Omicron XBB.1.5/1.9*
34	23688		La Spezia	La Spezia	3,64E+04		
92	23718		Genova	Recco	2,55E+05	Package A	Omicron XBB.1.5/1.9*
36	23735		Bergamo	Bergamo	5,28E+04		
37	23737		Cremona	Città di Cremona	4,88E+04		
38	23738		Brescia	Verziano	9,42E+04		
96	24634		Sondrio	Sondrio	2,22E+04		
97	24079		Milano	Milano San Rocco	<LOD		
98	24080	Lombardia	Como	Como	1,84E+05		
99	24081		Pavia	Pavia	<LOD		
100	24082		Como - Lecco - Milano - Monza e della Brianza	Monza	3,20E+05		
101	24083		Pavia	Vigevano	3,21E+03		
39	23782		Pesaro-Urbino	Borgheria	1,81E+05		
40	23783		Pesaro-Urbino	Ponte Metauro	6,88E+04		
41	23784	Marche	Ancona	Zipa	1,27E+05	Package D	Omicron JN.1*
42	23785		Ancona	Falconara	8,51E+04		

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82	23869		Termoli	Porto	1,73E+03	Package B[a]	Omicron XBB.1.5/1.9*
83	23870	Molise	Termoli	Pantano basso	1,03E+03	Package A	Omicron XBB.1.5/1.9*
84	23868		Campobasso	S.Pietro	4,67E+02		
43	23776		Bolzano	IDA Bolzano	1,17E+05	Package A	Omicron XBB.1.5/1.9*
44	23777	A.P. Bolzano	Bolzano	IDA Merano	2,09E+05	Package A (partial sequence, from V213E to N460K)[c]	Omicron XBB.1.5/1.9*
45	23778		Bolzano	IDA Termeno	1,84E+05	Package A (partial sequence, from V213E to N460K)[c]	Omicron XBB.1.5/1.9*
46	23667		Trento	Trento nord	1,17E+06	Package D	Omicron JN.1*
47	23668	A.P. Trento	Trento	Trento sud	9,52E+05		
48	23669		Trento	Rovereto	8,58E+05		
49	23605		Torino	Castiglione Torinese	4,43E+04		
50	23606		Biella	Biella Nord	1,24E+04		
51	23607		Biella	Biella Sud	7,78E+03		
52	23608	Piemonte	Novara	Novara	9,45E+03		
53	23654		Alessandria	Alessandria	1,05E+04		
54	23655		Asti	Asti	1,77E+04		
55	23656		Cuneo	Cuneo	4,22E+04		
56	23643		Bari	Bari Est	1,71E+03		
57	23644		Bari	Bari Ovest	4,26E+03		
58	23645	Puglia	Taranto	Taranto Bellavista	2,77E+03	Package B[a] + F157L + L452R	Omicron XBB.1.5/1.9*
59	23646		Taranto	Taranto Gennarini	2,89E+03		
60	23647		Trapani	Trapani	3,95E+03		
61	23648		Trapani	Mazara del Vallo	2,36E+03		
62	23649		Trapani	Marsala	2,94E+03		
63	23657		Ragusa	Modica	5,70E+03		
64	23658		Ragusa	Vittoria	2,98E+03		
65	23659	Sicilia	Ragusa	Ragusa	6,73E+03		
66	23660		Caltanissetta	Gela Macchitella	5,38E+03		
67	23766		Palermo	Acqua dei Corsari	2,23E+04	Package A+ L455F	Omicron XBB.1.5/1.9*
68	23767		Palermo	Fondo Verde	1,84E+04		
69	23768		Caltanissetta	Caltanissetta e San Cataldo	7,13E+04	Package B[a]	Omicron XBB.1.5/1.9*

85	23745		Catania	Pantano d'Arci	1,28E+04		
86	23746		Catania	Giarre	4,34E+04	M.E.[b]	Not assigned
87	23747		Siracusa	Siracusa	3,18E+04		
88	23744		Siracusa	Siracusa	3,32E+03		
106	24241	Toscana	Pisa	Pisa	7,04E+03		
107	24242		Lucca	Lucca	9,31E+03		
70	23638	Umbria	Perugia	Perugia - Pian della Genna	1,90E+05	Package D	Omicron JN.1*
71	23634		Padova	Padova Ca' Nordio - centro storico	9,14E+04		
72	23635		Padova	Padova Ca' Nordio - zip	1,29E+05	Package C	Omicron XBB.1.16*
73	23636		Padova	Padova Guizza	2,48E+05	Package A	Omicron XBB.1.5/1.9*
74	23637		Padova	Abano Terme	1,47E+05	Package A (partial sequence, from G339H to N460K)[c]	Omicron XBB.1.5/1.9*
75	23650	Veneto	Treviso	Treviso	4,91E+03	Package D (partial sequence, from L213G to N460K)[c]	Omicron JN.1*
76	23651		Vicenza	Vicenza Casale	1,38E+03		
77	23652		Venezia	Venezia Fusina	4,03E+03		
93	23574		Verona	Verona_collettore 8M	1,22E+04	Package A	Omicron XBB.1.5/1.9*
94	23575		Verona	Verona_collettore 3M	8,21E+02	M.E.[b]	Not assigned
95	23573		Verona	Verona_collettore 1M	8,94E+03	Package A + L455F + F456L	Omicron XBB.1.5/1.9*

NA= not available

[a] Package B may indicate the presence of the Omicron EG.5 sublineage. This last sublineage is characterised by the presence of the 'Q52H' mutation in approximately 93.2% and the 'F456L' mutation in about 96.2% of its sequences (https://outbreak.info/situation-reports, date: 26/01/2024).

[b] M.E. mixed electropherograms (noisy sequencing signal)

[c] Partial sequence due to mixed electropherograms and/or high signal noise; within brackets the region for which a sequence was provided.

Table 2. Sanger sequencing results

ID SAMPLES	Q52H	DEL69/70	V83A	V127F	G142D	DEL 144	H146Q/H146K	F157S	R158G	E180V	Q183E	DEL211/212	V213G	V213E	L216F	H245N	G252V	A264D	I332V	G339H	R346T	K356T	L368I	S371F	S373P	S375F	T376A	R403K	D405N	R408S	K417N	N440K	V445H	V445P	G446S	N450D	L452W	L455S	F456L	N460K	VARIANTS
28, 33, 43, 44, 45, 67, 73, 74, 83, 92, 93, 95			■		■	■	■				■			■			■			■	■		■	■	■	■	■		■	■	■	■		■	■					■	**Package A (Omicron XBB.1.5*/XBB.1.9*)**
1, 2, 7, 14, 16, 19, 22, 25, 29, 58, 69, 78, 82	■		■		■	■	■				■			■			■			■	■		■	■	■	■	■		■	■	■	■		■	■				■	■	**Package B (Omicron XBB.1.5*/XBB.1.9*)**
72			■		■	■	■			■	■			■			■			■	■		■	■	■	■	■		■	■	■	■		■	■					■	**Package C (Omicron XBB.1.16*)**
18, 23, 24, 26, 27, 30, 41, 46, 70, 75, 81		■	■	■	■	■		■	■		■	■	■		■		■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	**Package D (Omicron JN.1*)**

Limitations of the study

The geographical and population coverage of this flash survey is not representative of the entire territory of the country as it only covers 16 out of 21 of the Italian regions/Autonomous Provinces. It is important to highlight that the employment of molecular analytical methods in complex environmental matrices such as wastewater can be challenging due to a number of factors. These include low virus concentration, insufficient analytical recovery and/or PCR inhibitors. Consequently, both the detection/quantification and the PCR amplification required for the sequencing may produce false negatives, making molecular characterization and variant detection achievement difficult for all samples. In addition, obtainment of partial sequences from the spike region does not provide conclusive results for sublineage assignment. Our decision to adopt a broader lineage classification from 'outbreak.info' for variant classification, rather than specifying sublineage assignments, was influenced by the rapid evolution of numerous sublineages, often with minor differences, that hampered the reliable assignation to sublineages based solely on mutations observed in the spike region.

Conclusions and final considerations

This report is part of a monthly series focusing on SARS-CoV-2 and its variants in wastewater samples in Italy, in accordance with the EU Commission Recommendation 2021/472. The primary objective is to provide additional information on SARS-CoV-2 variants in the population, complementing data obtained through clinical surveillance. The results of this survey indicate that the Omicron variant is the sole SARS-CoV-2 variant in Italy, with the Omicron XBB.1.5*/XBB.1.9* lineages being the most prevalent. The Omicron JN.1* lineage and the Omicron XBB.1.16* lineage have also been detected. The sequencing of SARS-CoV-2 in wastewater samples provides valuable additional information alongside the sequencing of clinical cases. This approach provides a more complete and accurate understanding of the circulating variants in the country, contributing to a better characterization of the spread and evolution of this virus.

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